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(54) Title: MEANS AND METHODS FOR MODIFYING GENE EXPRESSION USING UNPOLYADENYLATED RNA

(57) Abstract: Methods and means are provided for reducing expression of a nucleic acid of interest, thereby altering the phenotype of an organism, particularly a plant, by providing aberrant, preferably unpolyadenylated, target specific RNA to the nucleus of the host cell. Preferably, the unpolyadenylated, target-specific RNA is provided by expression of a chimeric DNA construct comprising a promoter, a DNA region encoding the target specific RNA, a self-splicing ribozyme and a DNA region involved in 3' end formation and polyadenylation.

## MEANS AND METHODS FOR MODIFYING GENE EXPRESSION USING UNPOLYADENYLATED RNA

**Field of the invention.**

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The invention relates to methods for reducing the phenotypic expression of a nucleic acid of interest in plant cells by providing aberrant RNA molecules, preferably unpolyadenylated RNA molecules comprising at least one target specific nucleotide sequence homologous to the nucleic acid of interest, preferably a sense strand, into the nucleus of plant cells. The target-specific unpolyadenylated RNA molecules may be provided by introduction of chimeric DNAs which when transcribed under control of conventional promoter and 3' end formation and polyadenylation regions yield RNA molecules wherein at least the polyadenylation signal may be removed by the autocatalytic activity of a self-splicing ribozyme comprised within the transcribed RNA molecules. Also provided are plant cells comprising such RNA molecules or chimeric DNA encoding such RNA molecules, as well as plants. Similar methods and means for reducing the phenotypic expression of a nucleic acid by co-suppression in eukaryotic cells are provided.

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**Background of the invention.**

Post-transcriptional gene silencing (PTGS) or co-suppression, is a common phenomenon associated with transgenes in transgenic plants. PTGS results in sequence-specific removal of the silenced transgene RNA as well as homologous endogenous gene RNA or viral RNA. It is characterized by low steady-state mRNA levels with normal (usually high) rates of nuclear transcription of transgenes being maintained. There are a number of common features or characteristics for PTGS. PTGS is

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- i) sequence-specific;
- ii) systemically transmissible;
- iii) often associated with the presence of multiple copies of transgenes or with the use of strong promoters;
- 5 iv) frequently correlated with the presence of repetitive DNA structures, including inverted repeat T-DNA insertion patterns;
- v) often accompanied by de novo DNA methylation in the transcribed region, and
- vi) may be meiotically reset.

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A number of hypothetical models have been proposed to explain PTGS (see e.g. Wassenegger and Pélissier, 1998). Typically, these models suggest the involvement of a host encoded enzyme (RNA-directed RNA polymerase (RdRP)) which is proposed to use aberrant RNA as templates to synthesize  
15 small copy RNA molecules (cRNA). These cRNAs would then hybridize with the target mRNA to form duplex structures, thereby rendering the mRNA susceptible to degradation by endoribonucleases. So far, there has been no direct evidence that RdRP is involved in PTGS in plants.

20 An important question arising from the existing models is what type of RNA is the aberrant RNA that would be used as a template by RdRP, and in which cellular compartment RdRP would function.

Several reports have described the accumulation of unproductive or  
25 unpolyadenylated transgene RNA in plants which are post-transcriptionally silenced (Lee *et al.* 1997; van Eldik *et al.* 1998; Covey *et al.*, 1997; van Houdt *et al.*, 1997; Metzlaff *et al.* ; 1997).

The following documents relate to methods and means for regulating or  
30 inhibiting gene expression in a cell.

US 5,190,131 and EP 0 467 349 A1 describe methods and means to regulate or inhibit gene expression in a cell by incorporating into or associating with the genetic material of the cell a non-native nucleic acid sequence which is transcribed to produce an mRNA which is complementary to and capable of binding to the mRNA produced by the genetic material of that cell.

EP 0 223 399 A1 describes methods to effect useful somatic changes in plants by causing the transcription in the plant cells of negative RNA strands which are substantially complementary to a target RNA strand. The target RNA strand can be a mRNA transcript created in gene expression, a viral RNA, or other RNA present in the plant cells. The negative RNA strand is complementary to at least a portion of the target RNA strand to inhibit its activity *in vivo*.

EP 0 240 208 describes a method to regulate expression of genes encoded for in plant cell genomes, achieved by integration of a gene under the transcriptional control of a promoter which is functional in the host and in which the transcribed strand of DNA is complementary to the strand of DNA that is transcribed from the endogenous gene(s) one wishes to regulate.

EP 0 647 715 A1 and US patents 5, 034,323, 5,231,020 and 5,283,184 describe methods and means for producing plants exhibiting desired phenotypic traits, by selecting transgenotes that comprise a DNA segment operably linked to a promoter, wherein transcription products of the segment are substantially homologous to corresponding transcripts of endogenous genes, particularly endogenous flavonoid biosynthetic pathway genes.

Waterhouse *et al.* 1998 describe that virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and anti-sense

RNA. The sense and antisense RNA may be located in one transcript that has self-complementarity.

Hamilton *et al.* 1998 describes that a transgene with repeated DNA, i.e. inverted  
5 copies of its 5' untranslated region, causes high frequency, post-transcriptional suppression of ACC-oxidase expression in tomato.

WO 98/53083 describes constructs and methods for enhancing the inhibition of  
a target gene within an organism which involve inserting into the gene silencing  
10 vector an inverted repeat sequence of all or part of a polynucleotide region within the vector.

WO 95/34688 describes methods for cytoplasmic inhibition of gene expression  
and provides genetic constructs for the expression of inhibitory RNA in the  
15 cytoplasm of eukaryotic cells. The inhibitory RNA may be an anti-sense or a co-suppressor RNA. The genetic constructs are capable of replicating in the cytoplasm of a eukaryotic cell and comprise a promoter region, which may be a plant virus subgenomic promoter in functional combination with the RNA encoding region.

20 WO95/15394 and US 5908779 describe a method and construct for regulating gene expression through inhibition by nuclear antisense RNA in (mouse) cells. The construct comprises a promoter, antisense sequences, and a cis-or trans-ribozyme which generates 3'-ends independently of the polyadenylation  
25 machinery and thereby inhibits the transport of the RNA molecule to the cytoplasm.

**Summary of the invention.**

The present invention provides a method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, the method comprising the step of providing to the nucleus of that plant cell aberrant RNA comprising a target-specific nucleotide sequence, preferably unpolyadenylated RNA comprising a target specific nucleotide sequence, particularly by producing aberrant RNA such as unpolyadenylated RNA by transcription of a chimeric DNA comprised within the plant cell, the chimeric DNA comprising a plant-expressible promoter operably linked to a target specific DNA region encoding that RNA and optionally further comprising a DNA region involved in 3' end formation and polyadenylation, preceded by a self-splicing ribozyme encoding DNA region.

The invention also provides a method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, the method comprising the step of introducing into the nuclear genome of the plant cell a chimeric DNA to generate a transgenic plant cell, the chimeric DNA comprising the following operably linked parts:

- a) a plant-expressible promoter region, preferably a constitutive promoter or an inducible promoter or a tissue-specific promoter;
- b) a target-specific DNA region encoding a target-specific nucleotide sequence, preferably a target-specific DNA region comprising a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to the nucleic acid of interest or comprising a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to the complement of said nucleic acid of interest;
- c) a DNA region encoding a self-splicing ribozyme, preferably a self-splicing ribozyme comprising a cDNA copy of a self-splicing ribozyme from avocado sunblotch viroid, peach latent mosaic viroid, Chrysanthemum chlorotic mottle viroid, carnation stunt associated viroid, Newt satellite 2 transcript,

- Neurospora VS RNA, barley yellow dwarf virus satellite RNA, arabis mosaic virus satellite RNA, chicory yellow mottle virus satellite RNA S1, lucerne transient streak virus satellite RNA, tobacco ringspot virus satellite RNA, subterranean clover mottle virus satellite RNA, solanum nodiflorum mottle virus satellite RNA, velvet tobacco mottle virus satellite RNA, Cherry small circular viroid-like RNA or hepatitis delta virus RNA, particularly a DNA region comprising the nucleotide sequence of SEQ ID No 1 or SEQ ID No 2 or a ribozyme-effective part thereof; and
- 5 d) a DNA region involved in 3' end formation and polyadenylation;
- 10 wherein said chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.
- 15 Optionally, a transgenic plant may be regenerated from the transgenic plant cell. Preferably, the DNA region encoding a self-splicing ribozyme is located immediately upstream of the DNA region involved in 3' end formation and polyadenylation.
- 20 It is another objective of the invention to provide a chimeric DNA molecule for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, comprising
- a) a plant-expressible promoter region, preferably a constitutive promoter or an inducible promoter or a tissue-specific promoter;
- 25 b) a target-specific DNA region encoding a target-specific nucleotide sequence, preferably a target-specific DNA region comprising a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to the nucleic acid of interest or comprising a nucleotide sequence of at least 10 consecutive nucleotides

having at least about 70 % sequence identity to about 100 % sequence identity to the complement of said nucleic acid of interest;

- 5 c) a DNA region encoding a self-splicing ribozyme, preferably a self-splicing ribozyme comprising a cDNA copy of a self-splicing ribozyme from avocado sunblotch viroid, peach latent mosaic viroid, Chrysanthemum chlorotic mottle viroid, carnation stunt associated viroid, Newt satellite 2 transcript, Neurospora VS RNA, barley yellow dwarf virus satellite RNA, arabis mosaic virus satellite RNA, chicory yellow mottle virus satellite RNA S1, lucerne transient streak virus satellite RNA, tobacco ringspot virus satellite RNA, 10 subterranean clover mottle virus satellite RNA, solanum nodiflorum mottle virus satellite RNA, velvet tobacco mottle virus satellite RNA, Cherry small circular viroid-like RNA or hepatitis delta virus RNA, particularly a DNA region comprising the nucleotide sequence of SEQ ID No 1 or SEQ ID No 2 or a ribozyme-effective part thereof; and
- 15 d) a DNA region involved in 3' end formation and polyadenylation; wherein said chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first 20 RNA molecule comprising the polyadenylation site has been removed. Preferably, the DNA region encoding a self-splicing ribozyme is located immediately upstream of the DNA region involved in 3' end formation and polyadenylation.
- 25 It is yet another objective of the invention to provide plant cells and plants comprising a nucleic acid of interest which is normally capable of being phenotypically expressed, further comprising a chimeric DNA, preferably stably integrated into the nuclear genome, comprising
- 30 a) a plant-expressible promoter region, preferably a constitutive promoter or an inducible promoter or a tissue-specific promoter;



- b) a target-specific DNA region encoding a target-specific nucleotide sequence, preferably a target-specific DNA region comprising a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to the nucleic acid of interest or comprising a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to the complement of said nucleic acid of interest;
- c) a DNA region encoding a self-splicing ribozyme, preferably a self-splicing ribozyme comprising a cDNA copy of a self-splicing ribozyme from avocado sunblotch viroid, peach latent mosaic viroid, Chrysanthemum chlorotic mottle viroid, carnation stunt associated viroid, Newt satellite 2 transcript, Neurospora VS RNA, barley yellow dwarf virus satellite RNA, arabis mosaic virus satellite RNA, chicory yellow mottle virus satellite RNA S1, lucerne transient streak virus satellite RNA, tobacco ringspot virus satellite RNA, subterranean clover mottle virus satellite RNA, solanum nodiflorum mottle virus satellite RNA, velvet tobacco mottle virus satellite RNA, Cherry small circular viroid-like RNA or hepatitis delta virus RNA, particularly a DNA region comprising the nucleotide sequence of SEQ ID No 1 or SEQ ID No 2 or a ribozyme-effective part thereof; and
- d) a DNA region involved in 3' end formation and polyadenylation; wherein said chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.

The invention also provides a method for identifying a phenotype associated with the expression of a nucleic acid of interest in a plant cell, the method comprising:

- 1) selecting within the nucleic acid of interest a target sequence of at least 10 consecutive nucleotides;
  - 2) introducing a chimeric DNA into the nucleus of a suitable plant host cell comprising the nucleic acid of interest, the chimeric DNA comprising the following operably linked DNA fragments:
    - a) a plant-expressible promoter region;
    - b) a target-specific DNA region comprising a nucleotide sequence of at least about 70% to about 100% sequence identity to said target sequence or to the complement of said target sequence ; followed by
    - 10 c) a DNA region encoding a self-splicing ribozyme located immediately upstream of
    - d) a DNA region involved in 3' end formation and polyadenylation;
  - 3) observing the phenotype by a suitable method.
- 15 Yet another objective of the invention is to provide a method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a eukaryotic cell, the method comprising the step of providing to the nucleus of said eukaryotic cell aberrant RNA, preferably unpolyadenylated RNA, comprising a target specific nucleotide sequence of at
- 20 least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of the nucleic acid of interest, particularly by producing aberrant RNA such as unpolyadenylated RNA by transcription of a chimeric DNA comprised within the eukaryotic cell, the chimeric DNA comprising a plant-expressible promoter operably linked to a
- 25 target specific DNA region encoding that RNA and optionally further comprising a DNA region involved in 3' end formation and polyadenylation, preceded by a self-splicing ribozyme encoding DNA region.

Still another objective of the invention is to provide a method for reducing the

30 phenotypic expression of a nucleic acid of interest, which is normally capable of

being expressed in a eukaryotic cell, comprising the step of introducing into the nuclear genome of the eukaryotic cell a chimeric DNA to generate a transgenic plant cell, comprising the following operably linked parts:

- a) a promoter region functional in the eukaryotic cell;
- 5      b) a target-specific DNA region comprising nucleotide sequence of at least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of the nucleic acid of interest;
- c) a DNA region encoding a self-splicing ribozyme; and
- 10      d) a DNA region involved in 3' end formation and polyadenylation

wherein the chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first  
15      RNA molecule comprising the polyadenylation site has been removed.

The invention also provides a eukaryotic cell comprising a nucleic acid of interest, normally capable of being phenotypically expressed, further comprising a chimeric DNA comprising the following operably linked parts:

- 20      a) a promoter region functional in the eukaryotic cell;
- b) a target-specific DNA region comprising nucleotide sequence of at least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of the nucleic acid of interest;
- 25      c) a DNA region encoding a self-splicing ribozyme; and
- d) a DNA region involved in 3' end formation and polyadenylation

wherein said chimeric DNA when transcribed in the eukaryotic cell produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA  
30      molecule comprising a target specific nucleotide sequence wherein the 3' end of

the first RNA molecule comprising the polyadenylation site has been removed, as well as non-human eukaryotic organisms comprising or consisting essentially of such eukaryotic cells.

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### **Brief Description of the Figures**

Figure 1. Schematic representation of the ribozyme-containing GUS chimeric gene (pMBW267 and pMBW259) the control construct (pMBW 265) and the  
10 GUS chimeric gene used for supertransformation (pBPPGH). 35S-P: CaMV 35S promoter; GUS: region encoding  $\beta$ -glucuronidase; SAT: cDNA copy of the satellite RNA of Barley Yellow Dwarf Virus (BYDV) in positive strand orientation ( $\rightarrow$ ) or in minus strand orientation ( $\leftarrow$ ); Ocs-T: region from the octopine synthase gene from *Agrobacterium* involved in 3' end formation and polyadenylation; 3'  
15 Sat: cDNA copy of the 3' end of the satellite RNA of BYDV; 5' SAT: cDNA copy of the 5' end of the satellite RNA of BYDV; PP2-P: 1.3 kb promoter region of a gene encoding the cucurbit phloem protein PP2; Nos-T: region from the nopaline synthase gene from *Agrobacterium* involved in 3' end formation and polyadenylation; C: autocatalytic cleavage site in the RNA molecule transcribed  
20 from the chimeric gene.

### **Detailed description of the preferred embodiments of the invention.**

Although gene-silencing, either by anti-sense RNA or through co-suppression  
25 using sense RNA, is a commonly observed phenomenon in transgenic research, the intentional generation of gene-silenced transgenic eukaryotic cells and transgenic organisms, particularly plant cells and plants, still faces a number of problems. In particular the efficiency of gene-silencing is still amenable to improvement, both in number of transgenic lines exhibiting the phenomenon as

well as in the level of reduction of transcription and ultimately the phenotypic expression of particular nucleic acid of interest in a particular transgenic line.

A number of improved methods for gene-silencing have already been described, e.g. the simultaneous use in one cell of anti-sense and sense RNA targeted to  
5 the nucleic acid of interest, preferably co-located on one transcript exhibiting self-complementarity. Novel methods for increasing the efficiency of gene-silencing, preferably gene-silencing through co-suppression in a eukaryotic cell or organism, preferably plant cell or plant, and means therefore, are described in the different embodiments provided by the specification and claims.

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The current invention is based on the unexpected observation by the inventors, that the provision or the introduction of aberrant target-specific RNA, preferably unpolyadenylated target-specific RNA, particularly an aberrant target-specific RNA comprising a nucleotide sequence essentially identical to the nucleic acid  
15 of interest in sense orientation, into the nucleus of a cell of a eukaryotic organism, particularly a cell of plant, resulted in an efficient reduction of the expression of the nucleic acid of interest, both in the level of reduction as well as in the number of transgenic lines exhibiting gene-silencing. The understanding of hypothetical mechanisms through which gene-silencing, particularly PTGS, is  
20 supposed to proceed did not allow to predict that among all variables potentially involved in initiation and maintenance of gene-silencing, the selection of this one parameter-i.e. providing aberrant, preferably unpolyadenylated RNA- would have been sufficient to significantly increase the efficiency of gene-silencing, particularly gene-silencing through co-suppression.

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In one embodiment of the invention, a method is provided for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, comprising the step of providing aberrant RNA such as unpolyadenylated RNA which includes a target-specific nucleotide  
30 sequence to the nucleus of that plant cell. Conveniently, the aberrant RNA such

as unpolyadenylated RNA including the target-specific nucleotide sequence may be produced by transcription of a chimeric DNA or chimeric gene comprised within the plant cell, preferably incorporated, particularly stably integrated into the nuclear genome of the plant cell. In a particularly preferred embodiment, the  
5 aberrant RNA is unpolyadenylated RNA which still exhibits other modifications characteristic of mRNA, such as, but not limited to, the presence of a cap-structure at the 5' end.

As used herein, the term "expression of a gene" refers to the process wherein a  
10 DNA region which is operably linked to appropriate regulatory regions, particularly to a promoter region, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another nucleic acid or which is capable of being translated into a polypeptide or protein. A gene is said to encode an RNA when the end product of the expression of the gene is  
15 biologically active RNA, such as e.g. an antisense RNA, a ribozyme or a replicative intermediate. A gene is said to encode a protein when the end product of the expression of the gene is a protein or polypeptide.

A nucleic acid of interest is "capable of being expressed", when said nucleic  
20 acid, when introduced in a suitable host cell, particularly in a plant cell, can be transcribed (or replicated) to yield an RNA, and/or translated to yield a polypeptide or protein in that host cell.

The term "gene" means any DNA fragment comprising a DNA region (the  
25 "transcribed DNA region") that is transcribed into a RNA molecule (e.g., a mRNA) in a cell operably linked to suitable regulatory regions, e.g., a plant-expressible promoter. A gene may thus comprise several operably linked DNA fragments such as a promoter, a 5' leader sequence, a coding region, and a 3' region comprising a polyadenylation site. A plant gene endogenous to a  
30 particular plant species (endogenous plant gene) is a gene which is naturally

found in that plant species or which can be introduced in that plant species by conventional breeding. A chimeric gene is any gene which is not normally found in a plant species or, alternatively, any gene in which the promoter is not associated in nature with part or all of the transcribed DNA region or with at least  
5 one other regulatory region of the gene.

As used herein, "phenotypic expression of a nucleic acid of interest" refers to any quantitative trait associated with the molecular expression of a nucleic acid in a host cell and may thus include the quantity of RNA molecules transcribed or  
10 replicated, the quantity of post-transcriptionally modified RNA molecules, the quantity of translated peptides or proteins, the activity of such peptides or proteins.

A "phenotypic trait" associated with the phenotypic expression of a nucleic acid  
15 of interest refers to any quantitative or qualitative trait, including the trait mentioned, as well as the direct or indirect effect mediated upon the cell, or the organism containing that cell, by the presence of the RNA molecules, peptide or protein, or posttranslationally modified peptide or protein. The mere presence of a nucleic acid in a host cell, is not considered a phenotypic expression or a  
20 phenotypic trait of that nucleic acid, even though it can be quantitatively or qualitatively traced. Examples of direct or indirect effects mediated on cells or organisms are, e.g., agronomically or industrial useful traits, such as resistance to a pest or disease; higher or modified oil content etc.

25 As used herein, "reduction of phenotypic expression" refers to the comparison of the phenotypic expression of the nucleic acid of interest to the eukaryotic cell in the presence of the RNA or chimeric genes of the invention, to the phenotypic expression of the nucleic acid of interest in the absence of the RNA or chimeric genes of the invention. The phenotypic expression in the presence of the  
30 chimeric RNA of the invention should thus be lower than the phenotypic

expression in absence thereof, preferably be only about 25%, particularly only about 10%, more particularly only about 5% of the phenotypic expression in absence of the chimeric RNA, especially the phenotypic expression should be completely inhibited for all practical purposes by the presence of the chimeric RNA or the chimeric gene encoding such an RNA.

A reduction of phenotypic expression of a nucleic acid where the phenotype is a qualitative trait means that in the presence of the chimeric RNA or gene of the invention, the phenotypic trait switches to a different discrete state when compared to a situation in which such RNA or gene is absent. A reduction of phenotypic expression of a nucleic acid may thus, a.o., be measured as a reduction in transcription of (part of) that nucleic acid, a reduction in translation of (part of) that nucleic acid or a reduction in the effect the presence of the transcribed RNA(s) or translated polypeptide(s) have on the eukaryotic cell or the organism, and will ultimately lead to altered phenotypic traits. It is clear that the reduction in phenotypic expression of a nucleic acid of interest, may be accompanied by or correlated to an increase in a phenotypic trait.

As used herein "a nucleic acid of interest" or a "target nucleic acid" refers to any particular RNA molecule or DNA sequence which may be present in a eukaryotic cell, particularly a plant cell.

As used herein "aberrant RNA" refers to polyribonucleotide molecules which have characteristic differing from mRNA molecules normally found in that cell. The different characteristics include but are not limited to the absence or removal of a 5' cap structure, presence of persistent introns e.g. introns which have been modified in their splice sites so as to prevent splicing, or the absence of the polyA tail normally found associated with the mRNA ("unpolyadenylated RNA").



The term "target-specific nucleotide sequence" as used herein, refers to a nucleotide sequence (either DNA or RNA nucleotide sequence depending on the context) which can reduce the expression of the target nucleic acid of interest by gene-silencing. Preferably, only the expression of the target nucleic acid or gene, or nucleic acids or genes comprising essentially similar nucleotide sequence is reduced.

Preferably the target-specific nucleotide sequence comprises a nucleotide sequence corresponding to the "sense" nucleotide sequence of the nucleic acid or gene of interest. In other words, a target-specific sense nucleotide sequence may be essentially similar to part of an RNA molecule transcribed or produced from the nucleic acid or gene of interest or to parts of the nucleic acid or gene of interest controlling the production of that transcribed or produced RNA molecule, when read in the same 5' to 3' direction as the transcribed or produced RNA molecule.

Preferably, the target specific nucleotide sequence corresponds to part of a nucleic acid region from which RNA is produced, particularly a region which is transcribed and translated. It is particularly preferred that the target sequence corresponds to one or more consecutive exons, more particularly is located within a single exon of a coding region. However, the target specific nucleotide sequence may also be corresponding to untranslated regions of the RNA molecule produced from the nucleic acid or gene of interest. Moreover, in the light of a recent publication by Mette et al. (1999), it is expected that the target specific nucleotide sequence may also correspond to the regions controlling the production or transcription of RNA from the nucleotide or gene of interest, such as the promoter region.

The length of the sense target-specific nucleotide sequence may vary from about 10 nucleotides (nt) up to a length equaling the length (in nucleotides) of

the target nucleic acid. Preferably the total length of the sense nucleotide sequence is at least 10 nt, preferably 15 nt, particularly at least about 50 nt, more particularly at least about 100 nt, especially at least about 150 nt, more especially at least about 200 nt, quite especially at least about 550 nt. It is expected that there is no upper limit to the total length of the sense nucleotide sequence, other than the total length of the target nucleic acid. However for practical reason (such as e.g. stability of the chimeric genes) it is expected that the length of the sense nucleotide sequence should not exceed 5000 nt, particularly should not exceed 2500 nt and could be limited to about 1000 nt.

It will be appreciated that the longer the total length of the sense nucleotide sequence is, the less stringent the requirements for sequence identity between the total sense nucleotide sequence and the corresponding sequence in the target nucleic acid or gene become. Preferably, the total sense nucleotide sequence should have a sequence identity of at least about 75% with the corresponding target sequence, particularly at least about 80 %, more particularly at least about 85%, quite particularly about 90%, especially about 95%, more especially about 100%, quite especially be identical to the corresponding part of the target nucleic acid. However, it is preferred that the sense nucleotide sequence always includes a sequence of about 10 consecutive nucleotides, particularly about 20 nt, more particularly about 50 nt, especially about 100 nt, quite especially about 150 nt with 100% sequence identity to the corresponding part of the target nucleic acid. Preferably, for calculating the sequence identity and designing the corresponding sense sequence, the number of gaps should be minimized, particularly for the shorter sense sequences.

As used herein, "sequence identity" with regard to nucleotide sequences (DNA or RNA), refers to the number of positions with identical nucleotides divided by the number of nucleotides in the shorter of the two sequences. The alignment of

the two nucleotide sequences is performed by the Wilbur and Lipmann algorithm (Wilbur and Lipmann, 1983) using a window-size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4. Computer-assisted analysis and interpretation of sequence data, including sequence alignment as described  
5 above, can, e.g., be conveniently performed using the programs of the Intelligenetics™ Suite (Intelligenetics Inc., CA). Sequences are indicated as "essentially similar" when such sequence have a sequence identity of at least about 75%, particularly at least about 80 %, more particularly at least about 85%, quite particularly about 90%, especially about 95%, more especially about  
10 100%, quite especially are identical. It is clear than when RNA sequences are said to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

15 It is expected however, that the target-specific nucleotide sequence may also comprise a nucleotide sequence corresponding to the "antisense" nucleotide sequence of the nucleic acid or gene of interest. In other words, a target-specific antisense nucleotide sequence may be essentially similar to the complement of part of an RNA molecule transcribed or produced from the nucleic acid or gene  
20 of interest or to the complement of parts of the nucleic acid or gene of interest controlling the production of that transcribed or produced RNA molecule, when read in the same 5' to 3' direction as the transcribed or produced RNA molecule.

The requirements for antisense target-specific nucleotide sequences with regard  
25 to length, similarity etc. are expected to be essentially similar as for sense target-specific nucleotide sequences as specified herein.

It will be clear to the person skilled in the art that the unpolyadenylated RNA molecule may comprise more than one target-specific nucleotide sequence and  
30 particularly that the unpolyadenylated RNA molecule may comprise sense and

antisense target-specific nucleotide sequences wherein the sense and antisense nucleotide sequences are essentially complementary to each other and capable of forming an artificial hairpin structure as described in Waterhouse *et al.*, 1998 or in PCT-application PCT/IB99/00606 (incorporated by reference).

5

It will also be clear that the unpolyadenylated RNA molecule may comprise one or more RNA stabilizing elements. As used herein, "an RNA stabilizing element" is a nucleotide sequence which when included into an RNA molecule prolongs the half-life time of that RNA molecule, i.e. protects it from being degraded.

10 Preferred RNA stabilizing elements include stable stem-loop sequences, such as the stem-loop sequences found in the mRNA encoded by the histone genes in mammalian cells, which are involved in conferring stability to the histone mRNA. An example of such a histone stem loop encoding sequence is included in SEQ ID No 7. Homologous sequences or functional equivalent sequences to  
15 the sequence of SEQ ID No 7, derived from other organisms, particularly plants may also be used to the same effect.

Inclusion of such an RNA stabilizing element in an unpolyadenylated RNA molecule, or of a nucleotide sequence encoding such an RNA stabilizing  
20 element in a chimeric gene encoding the unpolyadenylated RNA molecule may further enhance the efficiency of gene-silencing of the target gene.

As indicated above, introduction of target-specific unpolyadenylated RNA into the nucleus of a plant cell can conveniently be achieved by transcription of a  
25 chimeric DNA encoding RNA introduced into the nucleus, preferably stably integrated into the nuclear genome of a plant cell.

In a preferred embodiment of the invention, the target-specific unpolyadenylated RNA may be produced in the nucleus of a plant cell by transcription of a  
30 chimeric DNA encoding a first target-specific RNA, which may be further

processed by the action of a ribozyme also present, and preferably also encoded by a chimeric gene, in the plant cell to yield a second unpolyadenylated target-specific RNA. It will be clear for the person skilled in the art that the RNA processing need not be subsequently but can occur simultaneously. In a particularly preferred embodiment the ribozyme is a self-splicing ribozyme which is comprised within the generated target specific RNA transcript.

Thus, in a particularly preferred embodiment of the invention, a method is provided for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, the method comprising the step of introducing into the nuclear genome of the plant cell a chimeric DNA to generate a transgenic plant cell, the chimeric DNA comprising the following operably linked parts:

- (a) a plant-expressible promoter region;
- (b) a target-specific DNA region;
- (c) a DNA region encoding a self-splicing ribozyme; and
- (d) a DNA region involved in 3' end formation and polyadenylation wherein the chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.

The method may optionally further comprise the step of regenerating a the transgenic plant cell into a transgenic plant.

As used herein, "a ribozyme" is a catalytic RNA molecule that has the intrinsic ability to break and form covalent bonds in ribonucleic acids at specific sites in the absence of a cofactor other than a divalent cation.

As used herein a "self-splicing ribozyme" or "self-cleaving ribozyme" is a ribozyme capable of autocatalysis at a specific site within that ribozyme. Preferred self-splicing ribozymes are self-splicing ribozymes with a so-called hammerhead structure. However, it is expected that self-cleaving ribozymes with  
5 another conformation such as the hairpin self-cleaving structures encountered in the minus strand of replication intermediates of e.g. the nepoviruses can also be used to the same effect.

Particularly preferred self-splicing ribozymes are those involved in the replication  
10 of small circular plant pathogenic RNAs, such as but not limited to the self-splicing ribozyme from avocado sunblotch viroid, peach latent mosaic viroid, Chrysanthemum chlorotic mottle viroid, carnation stunt associated viroid, Newt satellite 2 transcript, Neurospora VS RNA, barley yellow dwarf virus satellite RNA, arabis mosaic virus satellite RNA, chicory yellow  
15 mottle virus satellite RNA S1, lucerne transient streak virus satellite RNA, tobacco ringspot virus satellite RNA, subterranean clover mottle virus satellite RNA, solanum nodiflorum mottle virus satellite RNA, velvet tobacco mottle virus satellite RNAvSCMoV or Cherry small circular viroid-like RNAcscRNA1. Table 1 lists different variant ribozymes suitable for the invention, as well as a reference  
20 to their nucleotide sequence.

The DNA regions encoding self-splicing ribozymes may be cDNA copies of part of the mentioned plant pathogenic RNAs comprising the ribozyme, or may be synthetic DNA. Also comprised are variants such as mutants including  
25 substitutions, deletions or insertions of nucleotides within the ribozyme nucleotide sequence in such a way that the autocatalytic capacity of the ribozymes is not substantially altered.

Preferably, the DNA region encoding the self-splicing ribozyme is located  
30 immediately upstream of the DNA region encoding the 3' end formation and

polyadenylation signal. However, having read the specification, the person skilled in the art will immediately realize that the DNA region encoding the self-splicing ribozyme may be comprised within the chimeric gene encoding the unpolyadenylated RNA at other locations, provided that a sufficiently large  
5 second RNA comprising a target-specific nucleotide wherein the polyadenylation site is removed may be generated.

It will be clear that when an RNA stabilizing element (or the DNA sequence encoding such RNA stabilizing element) is included, the RNA stabilizing element  
10 should also preferably immediately precede the DNA region encoding the self-splicing ribozyme. However the RNA stabilizing element may be included at other locations, provided that it will be located in the unpolyadenylated RNA upon processing by the ribozyme.

### Table 1. Different self-cleaving ribozymes

Accession	Host	Source	Year	Accession	Host	Source
Avocado sunblotch viroid	Symons	1981	Nucleic Acids Res. 9 6527-6537	JO2020	hammerhead	hammerhead
Avocado sunblotch viroid variant C-10	Rakowski and Symons	1989	Virology 173 352-358	M31100	hammerhead	hammerhead
Avocado sunblotch viroid variant B-1	Rakowski and Symons	1989	Virology 173 352-358	M31086	hammerhead	hammerhead
Avocado sunblotch viroid variant A-2	Rakowski and Symons	1989	Virology 173 352-358	M31085	hammerhead	hammerhead
Avocado sunblotch viroid variant B-2	Rakowski and Symons	1989	Virology 173 352-358	M31087	hammerhead	hammerhead
Avocado sunblotch viroid variant C-2	Rakowski and Symons	1989	Virology 173 352-358	M31092	hammerhead	hammerhead
Avocado sunblotch viroid variant B-3	Rakowski and Symons	1989	Virology 173 352-358	M31088	hammerhead	hammerhead
Avocado sunblotch viroid variant C-3	Rakowski and Symons	1989	Virology 173 352-358	M31093	hammerhead	hammerhead
Avocado sunblotch viroid variant B-4	Rakowski and Symons	1989	Virology 173 352-358	M31089	hammerhead	hammerhead
Avocado sunblotch viroid variant C-4	Rakowski and Symons	1989	Virology 173 352-358	M31094	hammerhead	hammerhead
Avocado sunblotch viroid variant B-5	Rakowski and Symons	1989	Virology 173 352-358	M31090	hammerhead	hammerhead
Avocado sunblotch viroid variant C-5	Rakowski and Symons	1989	Virology 173 352-358	M31095	hammerhead	hammerhead
Avocado sunblotch viroid variant B-6	Rakowski and Symons	1989	Virology 173 352-358	M31091	hammerhead	hammerhead
Avocado sunblotch viroid variant C-6	Rakowski and Symons	1989	Virology 173 352-358	M31098	hammerhead	hammerhead
Avocado sunblotch viroid variant C-7	Rakowski and Symons	1989	Virology 173 352-358	M31097	hammerhead	hammerhead
Avocado sunblotch viroid variant C-8	Rakowski and Symons	1989	Virology 173 352-358	M31098	hammerhead	hammerhead
Avocado sunblotch viroid variant C-9	Rakowski and Symons	1989	Virology 173 352-358	M31099	hammerhead	hammerhead
Avocado sunblotch viroid ASBVd-B	Semandik and Szychowski	1994	J. Gen Virol. 75 1543-1549	S74687	hammerhead	hammerhead
Avocado sunblotch viroid ASBVd-V	Semandik and Szychowski	1994	J. Gen Virol. 75 1543-1549	S73861	hammerhead	hammerhead
Peach latent mosaic viroid PLMVd.1	Hernandez and Flores	1992	Proc. Natl. Acad. Sci. 89 3711-3715	M83545	hammerhead	hammerhead
Peach latent mosaic viroid PLMVd.2	Hernandez and Flores	1992	Proc. Natl. Acad. Sci. 89 3711-3715		hammerhead	hammerhead
Peach latent mosaic viroid Peach-Italy	Schamliou et al.	1995	Acta Hort. 386 522-530		hammerhead	hammerhead
Peach latent mosaic viroid Cherry-Canada	Hadini et al.	1997	Plant Dis. 81, 154-158		hammerhead	hammerhead
Peach latent mosaic viroid variant gds2	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005294	hammerhead	hammerhead
Peach latent mosaic viroid variant gds21	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005295	hammerhead	hammerhead
Peach latent mosaic viroid variant gds15	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005296	hammerhead	hammerhead
Peach latent mosaic viroid variant gds23	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005297	hammerhead	hammerhead
Peach latent mosaic viroid variant gds18	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005298	hammerhead	hammerhead
Peach latent mosaic viroid variant gds1	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005299	hammerhead	hammerhead
Peach latent mosaic viroid variant gds3	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005300	hammerhead	hammerhead
Peach latent mosaic viroid variant gds19	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005301	hammerhead	hammerhead
Peach latent mosaic viroid variant gds13	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005302	hammerhead	hammerhead
Peach latent mosaic viroid variant gds6	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005303	hammerhead	hammerhead
Peach latent mosaic viroid variant gds16	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005304	hammerhead	hammerhead
Peach latent mosaic viroid variant esc8	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005305	hammerhead	hammerhead



Peach latent mosaic viroid variant esc16	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005306	hammerhead	hammerhead
Peach latent mosaic viroid variant esc5	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005307	hammerhead	hammerhead
Peach latent mosaic viroid variant esc12	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005308	hammerhead	hammerhead
Peach latent mosaic viroid variant esc 10	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005309	hammerhead	hammerhead
Peach latent mosaic viroid variant esc 14	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005310	hammerhead	hammerhead
Peach latent mosaic viroid variant is4b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005311	hammerhead	hammerhead
Peach latent mosaic viroid variant is16b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005312	hammerhead	hammerhead
Peach latent mosaic viroid variant is17b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005313	hammerhead	hammerhead
Peach latent mosaic viroid variant is1	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005314	hammerhead	hammerhead
Peach latent mosaic viroid variant is18b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005315	hammerhead	hammerhead
Peach latent mosaic viroid variant is11	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005316	hammerhead	hammerhead
Peach latent mosaic viroid variant is8	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005317	hammerhead	hammerhead
Peach latent mosaic viroid variant is19b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005318	hammerhead	hammerhead
Peach latent mosaic viroid variant is5b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005319	hammerhead	hammerhead
Peach latent mosaic viroid variant is11b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005320	hammerhead	hammerhead
Peach latent mosaic viroid variant is6b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005321	hammerhead	hammerhead
Peach latent mosaic viroid variant is14b	Ambros et al.	1998	J. Virol. 72 7397-7406	AJ005322	hammerhead	hammerhead
Crysanthemum chlorotic mottle viroid	Navarro and Flores	1997	Proc. Natl. Acad. Sci. 94 11262-11267	Y14700	hammerhead	hammerhead
Barley yellow dwarf virus satellite RNA	Miller et al.	1991	Virology 183 711-720	M63666	hammerhead	hammerhead
Arabidopsis mosaic virus satellite RNA	Kaper et al.	1988	Biochem. Biophys. Res. Com. 154 318-325	M21212	hammerhead	hairpin
Chicory yellow mottle virus satellite RNA S1	Rubino et al.	1990	J. Gen. Virol. 71 1897-1903	D00721	hammerhead	hairpin
Lucerne transient streak virus satellite RNA LTSV-N	Keese et al.	1983	FEBS Lett. 159 185-190	X01885	hammerhead	hammerhead
Lucerne transient streak virus satellite RNA LTSV-A	Keese et al.	1983	FEBS Lett. 159 185-190	X01884	hammerhead	hammerhead
Lucerne transient streak virus satellite RNA LTSV-C	Abouhalder and Paliwal	1988	J. Gen. Virology 69 2369-2373	D00341	hammerhead	hammerhead
Tobacco ringspot virus satellite RNA. 1	Buzayan et al.	1986	Virology 151, 186-199	M14879	hammerhead	hairpin
Tobacco ringspot virus satellite RNA. 2	Buzayan et al.	1987	Virology 160, 95-99	M17439	hammerhead	hairpin
Subterranean clover mottle virus satellite RNA. 1	Davies et al.	1990	Virology 177, 216-224	M33001	hammerhead	
Subterranean clover mottle virus satellite RNA. 2	Davies et al.	1990	Virology 177, 216-224	M33000	hammerhead	
Solanum nodiflorum mottle virus RNA	Haseloff and Symons	1982	Nucleic Acids Res. 10 3681-3691	J02386	hammerhead	
Velvet tobacco mottle virus circular viroid-like RNA-1	Haseloff and Symons	1982	Nucleic Acids Res. 10 3681-3691		hammerhead	
Velvet tobacco mottle virus circular viroid-like RNA-2	Haseloff and Symons	1982	Nucleic Acids Res. 10 3681-3691	J02439	hammerhead	
Cherry small circular viroid-like RNA	Di Serio et al.	1997	J. Virol. 71 6603-6610	Y12833	mod. hammerhead	mod.
Carnation small viroid-like RNA-1	Hernandez et al.	1992	Nucleic Acids Res. 20 6323-6329	X68034	hammerhead	hammerhead
Carnation small viroid-like RNA-2	Hernandez et al.	1992	Nucleic Acids Res. 20 6323-6329		hammerhead	hammerhead
Notophthalmus viridescens (Newt) satellite 2 transcript	Epstein et al.	1986	J. Cell. Biol. 103 1137-1144	X04478	hammerhead	hammerhead
Neurospora VS RNA	Saville and Collins	1990	Cell 61 685-686	M32974	VS RNA selfcleavage	

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Schistosoma satellite DNA	
Ferbeyre et al.	
1998	
Mol. Cell. Biol. 18 3860-3868	
AF036739	

The use of ribozymes in transgenic organisms to generate RNA molecules with 5' and or 3' termini of interest has been documented in the art. Rubio *et al.* 1999, describe broad-spectrum protection against Tombusviruses elicited by defective interfering (DI) RNAs in transgenic plants. To produce RNAs with authentic 5' and 3' termini identical to those of native DI RNA, the DI RNA sequence transcribed from a DNA cassette was flanked by ribozymes. Transgenic *Nicotiana benthamiana* plants were better protected than non-transgenic plants against infection by tomato bushy stunt virus and related tombusviruses. DI RNAs interfere drastically with virus accumulation through effective competition with the parental virus for transacting factors required for replication. Egli and Braus, 1994 describe uncoupling of mRNA 3' cleavage and polyadenylation by expression of a hammerhead ribozyme in yeast. Eckner *et al.* 1991 described that test gene transcripts which could obtain a mature histone 3' end by the RNA cleaving activity of a cis-acting ribozyme, thus circumventing the cellular 3' end processing machinery were found to be transport deficient and accumulated in the nuclear compartment. However, these documents in the art are not related to methods for inhibiting phenotypic expression by homology dependent gene-silencing, particularly by PTGS.

20

A particularly preferred self-splicing ribozyme is the ribozyme comprised with the Barley yellow dwarf virus (BYDV) satellite RNA, quite particularly the satellite RNA found in BYDV isolates of the RPV serotype.

25 It has been found that reduction of the phenotypic expression of the nucleic acid of interest using a chimeric gene according to the invention was most efficient using a cDNA copy of the ribozyme comprised within the minus strand of BYDV satellite RNA. Therefore, ribozymes which show an autocatalytic activity similar to the autocatalytic activity of the ribozyme comprised within the minus strand of

BYDV satellite RNA are especially suited for the methods of the invention. Autocatalytic activity of ribozymes can be compared with the autocatalytic activity of the (-) strand of BYDV satellite RNA as described by Miller *et al.* 1991.

- 5 The ribozyme motif within the (-) strand of BYDV satellite RNA has been identified as the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 194 to the nucleotide at position 236. The ribozyme motif within the (+) strand of BYDV satellite RNA has been identified as the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 310 to the nucleotide at position 322 followed by the nucleotide sequence of SEQ ID No. 2 from the nucleotide at position 1 to the nucleotide at position 89.

- It goes without saying that more than one DNA region encoding a ribozyme may be comprised within the chimeric gene. These ribozymes may be clustered, e.g. they may all be located the region immediately proceeding DNA region encoding the '3 end formation and polyadenylation signal.

- However, it is expected that more than one DNA region encoding a ribozyme may be comprised within the chimeric gene in such a way that upon self-cleavage more than one unpolyadenylated RNA molecules each comprising a target-specific nucleotide sequence is generated. Such a chimeric DNA could thus comprise:

- a) a plant expressible promoter
- b) a first target-specific DNA region
- 25 c) a DNA region encoding a first self-splicing ribozyme
- d) a second target-specific DNA region
- e) a DNA region encoding a second self-splicing ribozyme
- f) a DNA region encoding a 3' end formation and polyadenylation signal.

The first and second self-splicing ribozyme may be identical, essentially similar or different. Likewise, the first and second target-specific DNA region encoding the RNA with a target-specific nucleotide sequence may be identical, essentially similar or different.

- 5 For practical reasons, it is thought that the number of DNA regions encoding a ribozyme within a single chimeric gene should not exceed five.

In a preferred embodiment, the nucleic acid of interest, whose phenotypic expression is targeted to be reduced, is a gene incorporated in the genome of a eukaryotic cell, particularly a plant cell. It will be appreciated that the means and methods of the invention can be used for the reduction of phenotypic expression of a gene which belongs to the genome of the cell as naturally occurring, (an endogenous gene), as well as for the reduction of phenotypic expression of a gene which does not belong to the genome of the cell as naturally occurring, but has been introduced in that cell (a transgene). The transgene can be introduced stably or transiently, and can be integrated into the nuclear genome of the cell, or be present on a replicating vector, such as a viral vector.

- 20 In another preferred embodiment, the nucleic acid of interest, whose phenotypic expression is targeted to be reduced is a viral nucleic acid, particularly a viral RNA molecule, capable of infecting a eukaryotic cell, particularly a plant cell. In this case, the phenotype to be reduced is the replication of the virus, and ultimately, the disease symptoms caused by the infecting virus.

25

For the purpose of the invention, the term "plant-expressible promoter" means a promoter which is capable of driving transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell. A whole range of plant

expressible promoters, is available to direct the transcription of the chimeric genes of the invention. These include, but are not limited to strong promoters such as CaMV35S promoters (e.g., Harpster *et al.*, 1988). In the light of the existence of variant forms of the CaMV35S promoter, as known by the skilled  
5 artisan, the object of the invention can equally be achieved by employing these alternative CaMV35S promoters and variants. It is also clear that other plant-expressible promoters, particularly constitutive promoters, such as the opine synthase promoters of the *Agrobacterium* Ti- or Ri-plasmids, particularly a nopaline synthase promoter, or subterranean clover virus promoters can be  
10 used to obtain similar effects. Also contemplated by the invention are chimeric genes to reduce the phenotypic expression of a nucleic acid in a cell, which are under the control of single subunit bacteriophage RNA polymerase specific promoters, such as a T7 or a T3 specific promoter, provided that the host cells also comprise the corresponding RNA polymerase in an active form.

15

It is a further object of the invention, to provide methods for reducing the phenotypic expression of a nucleic acid in specific cells, particularly specific plant cells by placing the chimeric genes of the invention under control of tissue-specific or organ-specific promoters. Such tissue-specific or organ-  
20 specific promoters are well known in the art and include but are not limited to seed-specific promoters (e.g., WO89/03887), organ-primordia specific promoters (An *et al.*, 1996), stem-specific promoters (Keller *et al.*, 1988), leaf specific promoters (Hudspeth *et al.*, 1989), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller *et al.*, 1989), tuber-specific promoters (Keil *et al.*, 1989), vascular tissue specific  
25 promoters (Peleman *et al.*, 1989), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

In another embodiment of the invention, the expression of a chimeric gene to reduce the phenotypic expression of a target nucleic acid can be controlled at will by the application of an appropriate chemical inducer, by operably linking the transcribed DNA region of the chimeric genes of the invention to a promoter  
5 whose expression is induced by a chemical compound, such as the promoter of the gene disclosed in European Patent publication ("EP") 0332104, or the promoter of the gene disclosed in WO 90/08826.

It will be clear to the person skilled in the art that the same effect in reducing the  
10 phenotypic expression of a nucleic acid in a plant cell may be achieved using a trans-splicing ribozyme to remove at least the polyadenylation site from the RNA transcript of a chimeric gene comprising a plant expressible promoter, a target-specific DNA region and a DNA region encoding a 3' end termination and polyadenylation signal to generate unpolyadenylated RNA comprising a target-  
15 specific nucleotide sequence.

As used herein "a trans-splicing ribozyme" is an RNA molecule capable of catalyzing the breakage or formation of a covalent bond within another RNA molecule at a specific site.  
20 The trans-splicing ribozyme should be chosen or designed in such a way that it recognizes a specific site preceding, preferably immediately preceding the polyadenylation signal of the RNA transcript comprising a target-specific nucleotide sequence. Methods to design such trans-splicing ribozyme with endoribonuclease activity are known in the art (see e.g. Haselhoff and Gerlach,  
25 1988, WO 89/05852)

The DNA region encoding a trans-splicing ribozyme may be comprised within the chimeric gene encoding the target-specific RNA. Upon transcription of the chimeric gene an RNA molecule comprising the trans-splicing ribozyme and the

target-specific nucleotide sequence may then generated, wherein the trans-splicing ribozyme is capable of cleaving a specific site preceding the polyadenylation site of another similar RNA molecule, to generate unpolyadenylated target-specific RNA molecules.

5

The trans-splicing ribozyme may also be provided by expression of another chimeric gene encoding an RNA molecule comprising the trans-splicing ribozyme in the same plant cell, according to methods and means available in the art (see e.g. Vaish *et al.* 1998; Bramlage *et al.* 1998).

10

Alternative methods may exist to provide unpolyadenylated target-specific RNA to the nucleus of a plant cell. Such methods include e.g. transcription of a chimeric gene, integrated in the nuclear genome of a plant cell comprising a target-specific DNA region, by an DNA-dependent RNA polymerase different from RNA polymerase II, such that RNA transcripts are generated independent from the normal processing mRNA machinery (including intron-splicing, capping and polyadenylation). This can be achieved e.g. by operably linking the target-specific DNA region to a promoter region, recognized by a single subunit RNA polymerase from a bacteriophage, such as but not limited to the T7 polymerase, and a DNA region comprising a terminator for such a polymerase. In this case, the plant cell needs to be provided with a chimeric gene encoding the corresponding RNA polymerase. Providing unpolyadenylated target-specific RNA to the nucleus of a plant cell can also be achieved e.g. by operably linking the target-specific DNA region to a promoter region, recognized by a eukaryotic RNA polymerase I or III, and a DNA region comprising a terminator for such a polymerase. The means and methods for constructing such chimeric genes and plant cells are described in detail in WO 97/49814 (incorporated by reference). Another alternative to provide unpolyadenylated target-specific RNA to the nucleus of a plant cell may include transcription of a chimeric gene comprising a

25



target –specific DNA region operably linked to a plant-expressible promoter and linked to a DNA region comprising a 3' end formation signal but not a polyadenylation signal.

- 5 Although not intending to limit the invention to a specific mode of action, it is expected that the trigger of the homology-dependent gene-silencing mechanisms of the cell, particularly the co-suppression mechanism, is the accumulation of target-specific RNA into the nucleus of that cell. Providing unpolyadenylated RNA to the nucleus of the cell may be one mechanism of
- 10 causing accumulation of target-specific RNA in a nucleus of a cell, but other aberrations such as the absence of a cap-structure or the presence of persistent introns etc. may constitute alternative ways to cause the accumulation of target-specific RNA in the nucleus of a cell.
- 15 Moreover, it is expected that other aberrations in the target-specific RNA molecules in addition to the absence of the polyA tail, including the absence of a cap-structure, or the presence of persistent introns or the presence of abnormal secondary structures, particularly the presence of giant hairpin structures, may have a cumulative effect on the inhibition of the normal transit of the RNA from
- 20 the nucleus to the cytoplasm and hence have a cumulative or synergistic effect on the reduction of the phenotypic expression of a nucleic acid of interest.

- The recombinant DNA comprising the chimeric gene to reduce the phenotypic expression of a nucleic acid of interest in a host cell, may be accompanied by a
- 25 chimeric marker gene, particularly when the stable integration of the transgene in the genome of the host cell is envisioned. The chimeric marker gene can comprise a marker DNA that is operably linked at its 5' end to a promoter, functioning in the host cell of interest, particularly a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light

inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable colour to the transformed plant cell, such as the A1 gene (Meyer *et al.*, 1987), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0,242,246), or can provide antibiotic resistance to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

A recombinant DNA comprising a chimeric gene to reduce the phenotypic expression of a gene of interest, can be stably incorporated in the nuclear genome of a cell of a plant. Gene transfer can be carried out with a vector that is a disarmed Ti-plasmid, comprising a chimeric gene of the invention, and carried by *Agrobacterium*. This transformation can be carried out using the procedures described, for example, in EP 0 116 718.

Alternatively, any type of vector can be used to transform the plant cell, applying methods such as direct gene transfer (as described, for example, in EP 0 233 247), pollen-mediated transformation (as described, for example, in EP 0 270 356, WO85/01856 and US 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 0 067 553 and US 4,407,956), liposome-mediated transformation (as described, for example, in US 4,536,475), and the like.

25

Other methods, such as microprojectile bombardment as described for corn by Fromm *et al.* (1990) and Gordon-Kamm *et al.* (1990), are suitable as well. Cells of monocotyledonous plants, such as the major cereals, can also be transformed using wounded and/or enzyme-degraded compact embryogenic

tissue capable of forming compact embryogenic callus, or wounded and/or degraded immature embryos as described in WO92/09696. The resulting transformed plant cell can then be used to regenerate a transformed plant in a conventional manner.

5

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric gene for reduction of the phenotypic expression of a nucleic acid of interest of the invention in other varieties of the same or related plant species, or  
10 in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert.

The means and methods of the invention can also be used for the reduction of gene expression by co-suppression in eukaryotic cells and organisms.

15

In one embodiment the invention provides a method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a eukaryotic cell, comprising the step of providing unpolyadenylated RNA comprising a target specific sense nucleotide sequence  
20 of at least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of the nucleic acid of interest, to the nucleus of the eukaryotic cell.

In another embodiment, a method is provided for reducing the phenotypic  
25 expression of a nucleic acid of interest, which is normally capable of being expressed in a eukaryotic cell, comprising the step of introducing into the nuclear genome of the eukaryotic cell a chimeric DNA to generate a transgenic plant cell, DNA comprising the following operably linked parts:

(a) a promoter region functional in the eukaryotic cell;

- (b) a target-specific DNA region comprising nucleotide sequence of at least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of the nucleic acid of interest;
- 5 (c) a DNA region encoding a self-splicing ribozyme; and
- (d) a DNA region involved in 3' end formation and polyadenylation wherein the chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.
- 10
- 15 Different preferred embodiments and definitions described in connection with the reduction of gene expression by homology dependent gene silencing in plant cells and plants also apply *mutatis mutandis* to the means and methods described for reduction of gene expression by co-suppression in eukaryotic cells and organisms. As used herein "eukaryotic cells" comprise plant cells, animal
- 20 cells and human cells and cells from yeasts and fungi as well as cultures of such cells.

It is a further object of the invention to provide eukaryotic cells, preferably plant cells and organisms (preferably plants) comprising the chimeric genes for the reduction of the phenotypic expression of a target nucleic acid as described in the invention.

25

The methods and means of the invention can thus be used to reduce phenotypic expression of a nucleic acid in a eukaryotic cell or organism, particularly a plant

cell or plant, for obtaining shatter resistance (WO 97/13865), for obtaining modified flower colour patterns (EP 522 880, US 5,231,020), for obtaining nematode resistant plants (WO 92/21757, WO 93/10251, WO 94/17194), for delaying fruit ripening (WO 91/16440, WO 91/05865, WO 91/16426, WO 92/17596, WO 93/07275, WO 92/04456, US 5,545,366), for obtaining male sterility (WO 94/29465, WO89/10396, WO 92/18625), for reducing the presence of unwanted (secondary) metabolites in organisms, such as glucosinolates (WO97/16559) or chlorophyll content (EP 779 364) in plants, for modifying the profile of metabolites synthesized in a eukaryotic cell or organisms by metabolic engineering e.g. by reducing the expression of particular genes involved in carbohydrate metabolism (WO 92/11375, WO 92/11376, US 5, 365, 016, WO 95/07355) or lipid biosynthesis (WO 94/18337, US 5, 530, 192), for delaying senescence (WO 95/07993), for altering lignification in plants (WO 93/05159, WO 93/05160), for altering the fibre quality in cotton (US 5, 597, 718), for increasing bruising resistance in potatoes by reducing polyphenoloxidase (WO 94/03607), etc.

The methods of the invention will lead to better results and/or higher efficiencies when compared to the methods using conventional sense or antisense nucleotide sequences and it is believed that other sequence-specific mechanisms regulating the phenotypic expression of target nucleic acids might be involved and/or triggered by the presence of the double-stranded RNA molecules described in this specification.

A particular application for reduction of the phenotypic expression of a transgene in a plant cell, *inter alia*, by antisense or sense methods, has been described for the restoration of male fertility, the latter being obtained by introduction of a transgene comprising a male sterility DNA (WO 94/09143, WO 91/02069). The nucleic acid of interest is specifically the male sterility DNA.

Again, the processes and products described in this invention can be applied to these methods in order to arrive at a more efficient restoration of male fertility.

It will be appreciated that the methods and means described in the specification  
5 can also be applied in High Throughput Screening (HTS) methods, for the identification or confirmation of phenotypes associated with the expression of a nucleic acid sequence with hitherto unidentified function in a eukaryotic cell, particularly in a plant cell.

Such a method comprises the steps of:

- 10 1. selecting a target sequence within the nucleic acid sequence of interest with unidentified or non-confirmed function/phenotype when expressed. Preferably, if the nucleic acid has putative open reading frames, the target sequence should comprise at least part of one of these open reading frames. The length of the target nucleotide sequence may vary  
15 from about 10 nucleotides up to a length equalling the length (in nucleotides) of the nucleic acid of interest with unidentified function.
2. Introducing a chimeric DNA into the nucleus of a suitable host cell, comprising the nucleic acid of interest, wherein the chimeric DNA comprises a promoter region suitable for expression in the host cell, a DNA region  
20 encoding the target-specific nucleotide sequence, and a DNA region encoding a self-splicing ribozyme located immediately upstream of a DNA region involved in 3' end formation and polyadenylation.
3. observing the phenotype by a suitable method. Depending on  
25 the phenotype expected, it may be sufficient to observe or measure the phenotype in a single cell, but it may also be required to culture the cells to obtain an (organized) multicellular level, or even to regenerate a transgenic organism, particularly a transgenic plant.

It is also clear that the methods and means of the invention are suited for the reduction of the phenotypic expression of a nucleic acid in all plant cells of all plants, whether they are monocotyledonous or dicotyledonous plants, particularly crop plants such as but not limited to corn, rice, wheat, barley, sugarcane, cotton, oilseed rape, soybean, vegetables (including chicory, brassica vegetables, lettuce, tomato), tobacco, potato, sugarbeet but also plants used in horticulture, floriculture or forestry. The means and methods of the invention will be particularly suited for plants which have complex genomes, such as polyploid plants.

10

It is expected that the chimeric RNA molecules produced by transcription of the chimeric genes described herein, can spread systemically throughout a plant, and thus it is possible to reduce the phenotypic expression of a nucleic acid in cells of a non-transgenic scion of a plant grafted onto a transgenic stock comprising the chimeric genes of the invention (or vice versa) a method which may be important in horticulture, viticulture or in fruit production.

15

The following non-limiting Examples describe the construction of chimeric genes for the reduction of the phenotypic expression of a nucleic acid of interest in a eukaryotic cell and the use of such genes. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, *Current Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

20

25

Throughout the description and Examples, reference is made to the following sequences:

- SEQ ID No 1: cDNA copy of the (-) strand of BYDV RPV satellite RNA
- 5 SEQ ID No 2: cDNA copy of the (+) strand of BYDV RPV satellite RNA
- SEQ ID No 3: oligonucleotide for PCR amplification (SatPR1)
- SEQ ID No 4: oligonucleotide for PCR amplification (SatPR2)
- SEQ ID No 5: oligonucleotide for PCR amplification (SatPR3)
- SEQ ID No 6: oligonucleotide for PCR amplification (SatPR4)
- 10 SEQ ID No 7: nucleotide sequence encoding a histone stem from mammalian histone genes.

- The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should not be construed, however,
- 15 as limiting the broad scope of the invention.



## Examples

### Example 1 : Experimental procedures

#### 1.1 Chimeric DNA Constructs

##### 5 *Ribozyme-containing GUS gene constructs and a control construct*

The ribozyme sequences used are the plus strand or negative strand self-cleavage sequences of the satellite RNA of the barley yellow dwarf virus (BYDV) RPV serotype, which was isolated in CSIRO Plant Industry (SEQ ID 1 and 2 ; Miller et al., 1991).

- 10 The two ribozyme-containing GUS constructs (pMBW259 and pMBW267) and one control GUS construct (pMBW265) are schematically drawn in Figure 1. pMBW259 contains two plus strand cleavage sites, while pMBW267 contains the negative strand cleavage site.

- To make these constructs, a  $\beta$ -glucuronidase (GUS) gene sequence was  
15 modified to contain a NcoI site around the translational start ATG and cloned into pART7 (Gleave, 1992) at the XhoI/EcoRI sites, forming pMBW258. The full-length BYDV-RPV satellite sequence was amplified by PCR using primers SatPR1 (SEQ ID No. 3) and SatPR4 (SEQ ID No. 6), digested with BamHI and cloned into pMBW258 at the BamHI site, and the resulting 35S-GUS-Sat-ocs  
20 cassette was excised and cloned into pART27 (Gleave, 1992), forming pMBW265. The same full-length satellite sequence was inserted into the BamHI site of pMBW258 but in the antisense orientation, and the resulting 35S-GUS-asSat-ocs was cloned into pART27 to give rise to pMBW267.

- To make pMBW259, the 3' and 5' halves of the satellite RNA sequences were  
25 amplified by PCR using primer pairs SatPR3 (SEQ ID No. 5) and SatPR4 (SEQ ID No. 6) , and using SatPR1 (SEQ ID No. 3) and SatPR2 (SEQ ID No 4),

respectively. Fusion of the full-length sequence with the 3' half and the 5' half sequences were made through ligation between the EcoRV and HpaI ends of the three PCR fragments. This fusion mimics the natural multimeric forms of the satellite RNA, and therefore maintains the plus strand cleavage property of the native forms. The fusion sequence was cloned into pGEM-3Z (Promega) at the 5 SacI/PstI sites, excised with HindIII/EcoRI, blunted, and inserted into pART7 at the SmaI site, into which the GUS sequence described above was then cloned at the XhoI/EcoRI sites. The resulting 3SS-GUS-Sat-ocs was inserted into pART27 at the NotI site, forming pMBW259.

#### 10 *The super-transforming GUS construct*

The BamHI fragment was excised from pIG121 Hm (Hiei et al., 1994) and cloned into pART7. The GUS-nos sequence was then excised by AclI, blunted, and inserted into pBluescript at the HincII site. The 1.3 kb promoter region of a cucurbit phloem protein PP2 gene was excised with NotI/HindIII from a lambda clone CPPI.3 and cloned into the above Bluescript plasmid. The resulting PP2- 15 GUS-nos was excised with NotI/KpnI and inserted into pWBVec2 (Wang et al., 1998), giving rise to pBPPGH (Fig. 1).

### **1.2 Tobacco transformation**

20 *Nicotiana tabacum* cv. W38 was transformed and regenerated into whole plants essentially as described by Ellis et al. 1987. For constructs pMBW259, pMBW265 and pMBW267, 50 mg/L kanamycin was included in the media for selection of transformed tissue. For construct pBPPGH, 25 mg/L hygromycin B was used.

### **1.3 GUS assay**

25 GUS gene expression was assayed histochemically or fluorometrically according to Jefferson et al. 1987.

**Example 2 : GUS expression in transgenic tobacco transformed with a single type of the GUS constructs.**

Transgenic plants containing pMBW259 and pMBW267 showed very low levels  
5 of GUS expression, as judged by lack of, or faint blue, GUS staining. Plants  
transformed with pMBW265 showed more GUS expression than with pMBW259  
and pMBW267, but the level was much lower than plants transformed with  
pBPPGH. The best pMBW265 lines expressed 13.3% of the GUS activity by an  
average pBPPGH line.

10

**Example 3 : GUS expression in super-transformed lines containing pBPPGH and one of the three other constructs of Example 1.**

In order to promote silencing of a normal GUS gene by the presence of the  
15 ribozyme sequence near the 3' end of the GUS gene transcript, plants  
containing pMBW259, pMBW265 or pMBW267 and pBPPGH were constructed  
by re-transformation. Histochemical GUS assays of the super-transformants  
showed that the pMBW267 background gave substantially higher proportions of  
transformants than the pMBW259 or the pMBW265 background that showed  
20 low levels of GUS expression as indicated by the lack of strong and uniform blue  
staining. Super-transformants containing pBPPGH and pMBW265 showed the  
best GUS expression.

Table 2 shows the result of fluorometric GUS (MUG) assay of the super-  
25 transformants. The lines (E and F) containing pBPPGH and pMBW267 showed  
uniformly low GUS expression compared with the other lines. The best GUS  
expression came from the C lines which contain pBPPGH and pMBW265.

Among the three constructs tested, pMBW265 does not contain the full-length functional ribozyme sequences of the BYDV satellite RNA in a continuous stretch, and is therefore expected to produce mainly poly(A)+ RNA. pMBW259 contains two copies of the plus strand ribozyme sequence, and should give rise  
5 to RNA that have poly(A) tails removed by ribozyme cleavage. pMBW267 contain the negative strand ribozyme. The negative strand ribozyme was previously shown to be much (at least 10-fold) more efficient than the plus strand ribozyme (Miller et al., 1991), and therefore it is expected that pMBW267 produces poly(A)- RNA more efficiently. Our experiment showed that the super-  
10 transformed lines having the pMBW267 background expressed uniformly low levels of GUS activity in comparison with the lines having the pMBW259 or the pMBW265 background. The highest GUS expressing lines were from the pMBW265 background, which does not produce polyA- RNA.

Table 2. MUG assay of super-transformed tobacco lines\*.

Super-transformed lines	MUG Readings	Super-transformed lines	MUG Readings	Super-transformed lines	MUG Readings
A1	10.1	C1	8.84	E1	4.32
A2	15.8	C2	16.9	E2	3.15
A3	30.6	C3	17.9	E3	3.56
A4	47.3	C4	22.8	E4	3.31
A5	0.29	C5	11.7	E5	3.68
A6	10.3	C6	14.5	E6	5.02
A7	5.8	C7	44.0	E7	2.63
A8	13.15	C8	19.0	E8	10.27
A9	7.34	C9	29.8	E9	10.81
A10	9.76	C10	32.1	E10	13.1
A11	17.74	C11	37.1	E11	5.10
A12	34.8	C12	2.51	E12	2.86
A13	4.33	C13	14.5	E13	4.00
A14	3.41	C14	25.8	E14	16.8
A15	11.2	C15	7.20	E15	4.02
A16	2.04	C16	30.2	E16	1.29
A17	13.29	C17	9.70	E17	1.78
A18	14.6	C18	13.4	E18	3.57
A19	0.14	C19	19.3	E19	0.43
A20	17.2	C20	17.0	E20	11.8
A21	9.22	D1	6.01	F1	5.73
A22	17.3	D2	12.9	F2	5.10
B1	9.57	D3	0.19	F3	4.16
B2	44.7	D4	7.88	F4	4.69
B3	17.7	D5	1.24	F5	0
B4	1.25	D6	0.44	F6	1.93
B5	13.5	D7	14.1	F7	3.21
B6	11.4	D8	0.91	F8	2.77
B7	6.28	D9	5.49	F9	1.86
B8	24.8	D10	1.30	F10	3.27
B9	16.3	D11	15.1	F11	2.85
B10	9.72	D12	6.63	F12	3.25
B11	3.71	D13	12.2	F13	2.17
B12	0.08	D14	15.8	F14	2.84
B13	20.6	D15	1.32	F15	3.11
B14	11.9	D16	2.29	F16	2.06
B15	3.11	D17	3.59	F17	2.90
B16	8.25	D18	22.1	F18	3.75
B17	4.12	D19	13.0	F19	4.16
B18	6.04	D20	4.37	F20	2.49

\* A and B, from super-transformation of two independent pMBW259 lines with pBPPGH; C and D, from super-transformation of two independent pMBW265 lines with pBPPGH; E and F, from super-transformation of two independent pMBW267 lines with pBPPGH.

5

#### **Example 4. Additional chimeric DNA constructs**

Additional chimeric DNA constructs are made using conventional DNA cloning  
10 techniques and introduced in plants comprising the appropriate target genes

##### GUS silencing constructs type 1

Ribozyme containing GUS constructs similar to pMBW259 and pMBW267 (see  
15 Example 1) are adapted to include a nucleotide sequence encoding an RNA stabilizing element (histone stem form mammalian histone genes; SEQ ID No 7) between the nucleotide sequence derived from the GUS gene and upstream of the ribozyme encoding DNA region.

##### 20 GUS silencing constructs type 2

Ribozyme containing GUS constructs similar to pMBW259 and pMBW267 (see  
Example 1), but wherein the nucleotide sequence derived from the GUS gene  
are in antisense orientation (i.e. opposite to these homologous sequences in  
25 pMBW259 and pMBW267) are adapted to include a nucleotide sequence encoding an RNA stabilizing element (histone stem form mammalian histone genes; SEQ ID No 7) between the nucleotide sequence derived from the GUS gene and upstream of the ribozyme encoding DNA region.

##### 30 GUS silencing constructs type 3

Chimeric Gus silencing genes are constructed similar to the chimeric GUS silencing genes described in WO 99/53050 (particularly page 36) comprising an

additional DNA region encoding a ribozyme between the DNA region encoding the hairpin RNA and the DNA region encoding the transcription termination and polyadenylation. These constructs comprise the following elements

- a CaMV35S promoter (as described in Example 1)
- 5 • a nucleotide sequence of at least 500 bp derived from the GUS gene in sense orientation
- a spacer nucleotide sequence (e.g. comprising about 700 bp of the PVY Nia gene, see WO99/53050)
- the complement of the nucleotide sequence derived from the GUS gene (i.e. 10 part of the GUS gene in antisense orientation)
- a ribozyme encoding DNA region as in pMBW259 and pMBW267 (Example 1)
- an ocs-T terminator (as described in Example 1)

15 PVY resistance constructs

Chimeric PVY resistance genes are constructed comprising the following elements

- 20 • a CaMV35S promoter (as described in Example 1)
- a nucleotide sequence comprising about 700 bp of the PVY Nia gene, see WO99/53050) in sense orientation
- a spacer nucleotide sequence (e.g. part of the GUS gene)
- the complement of the nucleotide sequence derived from PVY (i.e. part of 25 the PVY sequence in antisense orientation)
- a ribozyme encoding DNA region as in pMBW259 and pMBW267 (Example 1)
- an ocs-T terminator (as described in Example 1)

30 When Gus silencing constructs are analysed, the transgenic plants comprise a functional GUS transgene and the silencing constructs are introduced either by

direct transformation of transgenic GUS gene containing plants or by crossing appropriate transgenic plants.

When PVY silencing constructs are used, transgenic plants comprising the PVY  
5 silencing constructs are inoculated with PVY, according to standard methods  
(see WO 99/53050).

In transgenic plants containing a GUS transgene, GUS expression is efficiently  
silenced upon introduction of the GUS silencing constructs in the majority of the  
10 obtained transgenic lines.

Transgenic plants containing the PVY resistance genes, are extremely resistant  
to infection by PVY in the majority of the obtained transgenic lines.



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What is claimed is:

- 1) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, said method  
5 comprising the step of providing to the nucleus of said plant cell aberrant RNA comprising a target specific nucleotide sequence.
- 2) The method of claim 1, wherein said aberrant RNA is unpolyadenylated RNA.  
10
- 3) The method of claim 2, wherein said unpolyadenylated RNA comprising said target specific nucleotide sequence is produced by transcription of a chimeric DNA comprised within said plant cell, said chimeric DNA comprising a plant-expressible promoter operably linked to a target specific DNA region  
15 encoding said RNA.
- 4) The method of claim 3, wherein said chimeric DNA further comprises a DNA region involved in 3' end formation and polyadenylation, preceded by a self-splicing ribozyme encoding DNA region.  
20
- 5) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, said method comprising the step of introducing into the nuclear genome of said plant cell a chimeric DNA to generate a transgenic plant cell, said chimeric DNA  
25 comprising the following operably linked parts:
  - a) a plant-expressible promoter region;
  - b) a target-specific DNA region;
  - c) a DNA region encoding a self-splicing ribozyme; and

- d) a DNA region involved in 3' end formation and polyadenylation wherein said chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.
- 5
- 6) The method of claim 5, wherein said DNA region encoding a self-splicing ribozyme is located immediately upstream of said DNA region involved in 3' end formation and polyadenylation.
- 10
- 7) The method of claim 5, wherein said DNA region encoding a self-splicing ribozyme comprises a cDNA copy of a self-splicing ribozyme from avocado sunblotch viroid, peach latent mosaic viroid, Chrysanthemum chlorotic mottle viroid, carnation stunt associated viroid, Newt satellite 2 transcript, Neurospora VS RNA, barley yellow dwarf virus satellite RNA, arabis mosaic virus satellite RNA, chicory yellow mottle virus satellite RNA S1, lucerne transient streak virus satellite RNA, tobacco ringspot virus satellite RNA, subterranean clover mottle virus satellite RNA, solanum nodiflorum mottle virus satellite RNA, velvet tobacco mottle virus satellite RNA, Cherry small circular viroid-like RNA or hepatitis delta virus RNA.
- 15
- 20
- 8) The method of claim 5, wherein said DNA region encoding a self-splicing ribozyme comprises a cDNA copy of a self-splicing ribozyme from barley yellow dwarf virus satellite RNA.
- 25
- 9) The method of claim 8, wherein said DNA region encoding a self-splicing ribozyme comprises the nucleotide sequence of SEQ ID No 1.

- 10)The method of claim 8, wherein said DNA region encoding a self-splicing ribozyme comprises the nucleotide sequence of SEQ ID No 2.
- 5 11)The method of claim 5, wherein said target-specific DNA region comprises a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to said nucleic acid of interest.
- 10 12)The method of claim 5, wherein said target-specific DNA region comprises a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to the complement of said nucleic acid of interest.
- 15 13)The method of claim 5, wherein said plant-expressible promoter is constitutive.
- 14)The method of claim 5, wherein said plant-expressible promoter is inducible.
- 20 15)The method of claim 5, wherein said plant-expressible promoter is tissue-specific.
- 16)The method of claim 5, wherein said nucleic acid of interest is a transgene.
- 25 17)The method of claim 5, wherein said nucleic acid of interest is an endogenous gene.
- 18)The method of claim 5, wherein said nucleic acid of interest is comprised within a virus or viral vector.

19)The method of claim 5, comprising the further step of regenerating a transgenic plant from said transgenic plant cell.

5 20)The method of claim 5, wherein said chimeric DNA further comprises a DNA region encoding an RNA stabilizing element, preceding said DNA region encoding a self-splicing ribozyme.

21)A chimeric DNA molecule for reducing the phenotypic expression of a nucleic  
10 acid of interest, which is normally capable of being expressed in a plant cell, said chimeric DNA molecule comprising  
a) a plant-expressible promoter region;  
b) a target-specific DNA region;  
c) a DNA region encoding a self-splicing ribozyme; and  
15 d) a DNA region involved in 3' end formation and polyadenylation;  
wherein said chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence  
20 wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.

22)The chimeric DNA molecule of claim 21, wherein said DNA region encoding a self-splicing ribozyme is located immediately upstream of said DNA region  
25 involved in 3' end formation and polyadenylation.

23)The chimeric DNA molecule of claim 21, wherein said DNA region encoding a self-splicing ribozyme comprises a cDNA copy of a self-splicing ribozyme from avocado sunblotch viroid, peach latent mosaic viroid, Chrysanthemum

chlorotic mottle viroid, carnation stunt associated viroid, Newt satellite 2 transcript, Neurospora VS RNA, barley yellow dwarf virus satellite RNA, arabis mosaic virus satellite RNA, chicory yellow mottle virus satellite RNA.S1, lucerne transient streak virus satellite RNA, tobacco ringspot virus satellite RNA, subterranean clover mottle virus satellite RNA, solanum nodiflorum mottle virus satellite RNA, velvet tobacco mottle virus satellite RNAvSCMoV or Cherry small circular viroid-like RNAscRNA1.

24)The chimeric DNA molecule of claim 21, wherein said DNA region encoding a self-splicing ribozyme comprises a cDNA copy of a self-splicing ribozyme from barley yellow dwarf virus satellite RNA.

25)The chimeric DNA molecule of claim 24, wherein said DNA region encoding a self-splicing ribozyme comprises the nucleotide sequence of SEQ ID No 1.

26)The chimeric DNA molecule of claim 24, wherein said DNA region encoding a self-splicing ribozyme comprises the nucleotide sequence of SEQ ID No 2.

27)The chimeric DNA molecule of claim 21, wherein said target-specific DNA region comprises a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to said nucleic acid of interest.

28)The chimeric DNA molecule of claim 21, wherein said target-specific DNA region comprises a nucleotide sequence of at least 10 consecutive nucleotides having at least about 70 % sequence identity to about 100 % sequence identity to the complement of said nucleic acid of interest.

29) The chimeric DNA molecule of claim 21, wherein said chimeric DNA molecule further comprises a DNA region encoding an RNA stabilizing element, preceding said DNA region encoding a self-splicing ribozyme.

5 30) A plant cell comprising a nucleic acid of interest which is normally capable of being phenotypically expressed, further comprising the chimeric DNA of any one of claims 21 to 29.

31) A plant comprising the plant cell of claim 30.

10

32) A method for identifying a phenotype associated with the expression of a nucleic acid of interest in a plant cell, said method comprising:

- a) selecting within said nucleic acid of interest a target sequence of at least 10 consecutive nucleotides;
- 15 b) introducing a chimeric DNA into the nucleus of a suitable plant host cell comprising said nucleic acid of interest, said chimeric DNA comprising the following operably linked DNA fragments:
  - i) a plant-expressible promoter region;
  - ii) a target-specific DNA region comprising a nucleotide sequence of at least about 70% to about 100% sequence identity to said target  
20 sequence or to the complement of said target sequence ; followed by
  - iii) a DNA region encoding a self-splicing ribozyme located immediately upstream of
  - iv) a DNA region involved in 3' end formation and polyadenylation;
- 25 c) observing the phenotype by a suitable method.

33) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a eukaryotic cell, said method comprising the step of providing to the nucleus of said eukaryotic cell



aberrant RNA comprising a target specific nucleotide sequence of at least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of said nucleic acid of interest.

5

34) The method of claim 33, wherein said aberrant RNA is unpolyadenylated RNA.

35) The method of claim 34, wherein said unpolyadenylated RNA comprising  
10 said target specific nucleotide sequence is produced by transcription of a chimeric DNA comprised within said eukaryotic cell, said chimeric DNA comprising a promoter functional in said eukaryotic cell, operably linked to a target specific DNA region encoding said RNA.

15 36) The method of claim 35, wherein said chimeric DNA further comprises a DNA region involved in 3' end formation and polyadenylation, preceded by a self-splicing ribozyme encoding DNA region.

20 37) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a eukaryotic cell, said method comprising the step of introducing into the nuclear genome of said eukaryotic cell a chimeric DNA to generate a transgenic plant cell, said chimeric DNA comprising the following operably linked parts:  
a) a promoter region functional in said eukaryotic cell;  
25 b) a target-specific DNA region comprising nucleotide sequence of at least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of said nucleic acid of interest;  
c) a DNA region encoding a self-splicing ribozyme; and

- d) a DNA region involved in 3' end formation and polyadenylation wherein said chimeric DNA when transcribed produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.

- 38) A eukaryotic cell comprising a nucleic acid of interest, normally capable of being phenotypically expressed, further comprising a chimeric DNA comprising the following operably linked parts:
- a) a promoter region functional in said eukaryotic cell;
  - b) a target-specific DNA region comprising nucleotide sequence of at least 10 consecutive nucleotides with at least about 70% sequence identity to about 100% sequence identity to the nucleotide sequence of said nucleic acid of interest;
  - c) a DNA region encoding a self-splicing ribozyme; and
  - d) a DNA region involved in 3' end formation and polyadenylation wherein said chimeric DNA when transcribed in said eukaryotic cell produces a first RNA molecule comprising a target specific nucleotide sequence and a self-splicing ribozyme, which when cleaved by autocatalysis produces a second RNA molecule comprising a target specific nucleotide sequence wherein the 3' end of the first RNA molecule comprising the polyadenylation site has been removed.

25

- 39) A non-human eukaryotic organism comprising the eukaryotic cell of claim 36.

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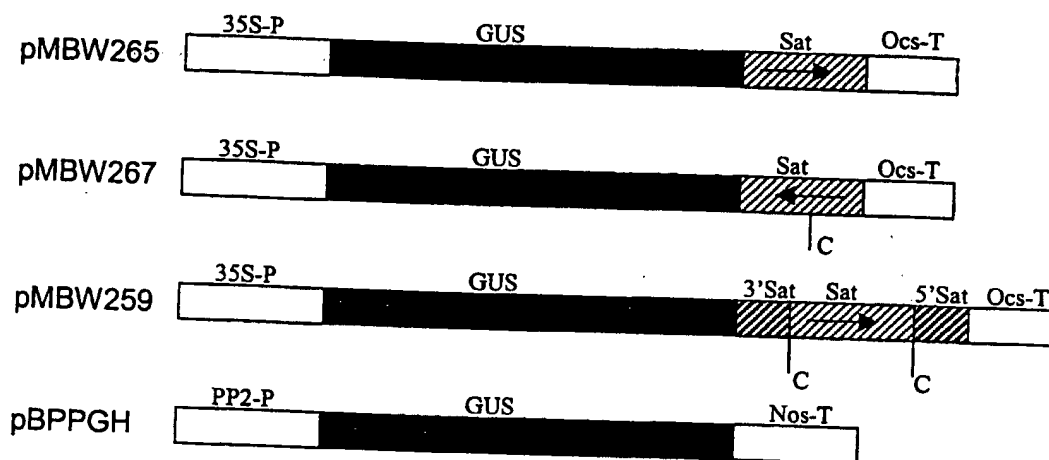


Figure 1

## SEQUENCE LISTING

<110> Wang, Ming-Bo  
 Waterhouse, Peter  
 Commonwealth Scientific and Industrial Research or

<120> Methods and means for obtaining modified phenotypes

<130> PLTPS

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<150> US 09/373720

<151> 1999-08-13

<160> 7

<170> PatentIn Ver. 2.1

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<220>

<223> Description of Artificial Sequence:nucleotide  
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histone gene

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21

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IB00/01133

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. <sup>7</sup>: C12N 15/63, 15/82

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
AS ABOVE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
AS BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Chem Abs, Medline, WPIDS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/15394 (UNIVERSITY OF CONNECTICUT) 8.6.99 Whole document	All
X	Liu, Zhong et al. Nuclear antisense RNA- an efficient new method to inhibit gene expression. Molecular Biotech. 1994. 2:107-118 Whole document	All
Y	Liu, Zhong et al. Targeted nuclear antisense RNA mimics natural antisense-induced degradation of polyoma virus early RNA. PNAS. 1994. 91: 4258-4262. Particularly Abstract, Materials and Methods and Discussion	All

☒ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 12 October 2000	Date of mailing of the international search report 16 OCT 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929	Authorized officer GILLIAN ALLEN Telephone No : (02) 6283 2266

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB00/01133

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Donahue C et al. Kinetics of hairpin ribozyme cleavage in yeast. RNA. 3: 961-973 Particularly pp 962, 968-9.	All
A	Welch, PJ. Expression of ribozymes in gene transfer systems to modulate target RNA levels. Curr Op Biol. 1998. 9: 486-496. Whole document	All



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB00/01133

## Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos : 1, 3, 34 and 35  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
The term "aberrant RNA" is considered to be so broad that these claims are not limited to the technical features of the invention, and no meaningful search can be carried out on the claims
  
3. ☐ Claims Nos :  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

## Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.  
**PCT/IB00/01133**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search  
Report

Patent Family Member

WO 95/15394

AU 12614/95

US 5908779

END OF ANNEX